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Multiple hybridization events in Cardamine (Brassicaceae) during the last 150 years: revisiting a textbook example of neoallopolyploidy

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Abstract: Background and Aims: Recently formed allopolyploid species represent excellent subjects for exploring early stages of polyploid evolution. The hexaploid *Cardamine schulzii* was regarded as one of the few nascent allopolyploid species formed within the past 150 years that presumably arose by autopolyploidization of a triploid hybrid, *C. × insueta*; however, the most recent investigations have shown that it is a trigenomic hybrid. The aims of this study were to explore the efficiency of progenitor-specific microsatellite markers in detecting the hybrid origins and genome composition of these two allopolyploids, to estimate the frequency of polyploid formation events, and to outline their evolutionary potential for long-term persistence and speciation. Methods: Flow-cytometric ploidy-level screening and genotyping by progenitor-specific microsatellite markers (20 microsatellite loci) were carried out on samples focused on hybridizing populations at Urnerboden, Switzerland, but also including comparative material of the parental species from other sites in the Alps and more distant areas. Key Results: It was confirmed that hybridization between the diploids *C. amara* and *C. rivularis* auct. gave rise to triploid *C. × insueta*, and it is inferred that this has occurred repeatedly. Evidence is provided that *C. schulzii* comprises three parental genomes and supports its origin from hybridization events between *C. × insueta* and the locally co-occurring hypotetraploid *C. pratensis*, leading to two cytotypes of *C. schulzii*: hypopentaploid and hypoheptaploid. Each cytotype of *C. schulzii* is genetically uniform, suggesting their single origins. Conclusions: Persistence of *C. schulzii* has presumably been achieved only by perennial growth and clonal reproduction. This contrasts with *C. × insueta*, in which multiple origins and occasional sexual reproduction have generated sufficient genetic variation for long-term survival and evolutionary success. This study illustrates a complex case of recurrent hybridization and polyploidization events, and highlights the role of triploids that promoted the origin of trigenomic hybrids.

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Multiple hybridization events in *Cardamine* (Brassicaceae) during the last 150 years: revisiting a textbook example of neoallopolyploidy

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- **Background and Aims** Recently formed allopolyploid species represent excellent subjects for exploring early stages of polyploid evolution. The hexaploid *Cardamine schulzii* was regarded as one of the few nascent allopolyploid species formed within the past ~150 years that presumably arose by autopolyploidization of a triploid hybrid, *C. × insueta*; however, the most recent investigations have shown that it is a trigonomic hybrid. The aims of this study were to explore the efficiency of progenitor-specific microsatellite markers in detecting the hybrid origins and genome composition of these two allopolyploids, to estimate the frequency of polyploid formation events, and to outline their evolutionary potential for long-term persistence and speciation.
- **Methods** Flow-cytometric ploidy-level screening and genotyping by progenitor-specific microsatellite markers (20 microsatellite loci) were carried out on samples focused on hybridizing populations at Urnerboden, Switzerland, but also including comparative material of the parental species from other sites in the Alps and more distant areas.
- **Key Results** It was confirmed that hybridization between the diploids *C. amara* and *C. rivularis* auct. gave rise to triploid *C. × insueta*, and it is inferred that this has occurred repeatedly. Evidence is provided that *C. schulzii* comprises three parental genomes and supports its origin from hybridization events between *C. × insueta* and the locally co-occurring hypotetraploid *C. pratensis*, leading to two cytotypes of *C. schulzii*: hypopentaploid and hypohexaploid. Each cytotype of *C. schulzii* is genetically uniform, suggesting their single origins.
- **Conclusions** Persistence of *C. schulzii* has presumably been achieved only by perennial growth and clonal reproduction. This contrasts with *C. × insueta*, in which multiple origins and occasional sexual reproduction have generated sufficient genetic variation for long-term survival and evolutionary success. This study illustrates a complex case of recurrent hybridization and polyploidization events, and highlights the role of triploids that promoted the origin of trigonomic hybrids.

Key words: Allopolyploidy, Alps, *Cardamine × insueta*, *Cardamine schulzii*, Brassicaceae, hybridization, microsatellites, polyploid evolution, speciation, neoallopolyploidy.

INTRODUCTION

The integrity of plant species is maintained by pre- and post-mating isolating mechanisms. They form barriers to interspecific gene flow, but these may be incomplete or may break down in certain circumstances. In fact, interspecific hybridization has been rather frequently reported in angiosperms, indicating that failure of isolating mechanisms is not rare (Arnold, 1997; Hegarty and Hiscock, 2005; Mallet, 2005; Rieseberg and Willis, 2007). Once hybridization occurs, it may have different outcomes, including hybrids that persist for a short time and reinforcement of barriers to gene exchange, the existence of highly variable hybrid swarms in contact zones, or new, stabilized hybrid (homoploid or allopolyploid) species (Urbanska and Landolt, 1998; Soltis and Soltis, 2009; Abbott *et al.*, 2013). Indeed, hybridization and polyploidization have been recognized as major speciation forces, contributing significantly to plant diversity (Otto and Whitton, 2000; Soltis and Soltis,

2009; Soltis *et al.*, 2009; Wood *et al.*, 2009). It is particularly challenging to identify the processes responsible for the origin, establishment and persistence of a polyploid/hybrid and to discern its multiple formation, subsequent genome rearrangements, transcriptome dynamics and ecological selection, as well as the repeatability of these processes (e.g. Adams and Wendel, 2005; Gross and Rieseberg, 2005; Hegarty and Hiscock, 2005, 2008; Doyle *et al.*, 2008; Soltis and Soltis, 2009). Such studies provide fascinating insights into the mechanisms of plant evolution and help us to understand plant species diversity.

A few allopolyploids inferred to have originated within the past ~100–200 years have become models for the study of the early stages of polyploid evolution (e.g. *Spartina anglica*, Ainouche *et al.*, 2004; *Senecio cambrensis*, Abbott and Lowe, 2004; *Tragopogon mirus* and *T. miscellus*, Soltis *et al.*, 2004; Symonds *et al.*, 2010). Although *Cardamine schulzii* has been listed among these few cases (Urbanska-Worytkiewicz, 1977a; Urbanska and Landolt, 1998; Soltis and Soltis, 2009), it has

received significantly less attention and many aspects remain unexplored.

Cardamine (Brassicaceae) is a species-rich (~200 species) genus of annual to perennial herbs distributed worldwide, characterized by high chromosome number variation and a high incidence of polyploidy (Lihová and Marhold, 2006; Carlsen et al., 2009). Both auto- and allopolyploidization have been fundamental for speciation in this genus, although the evolutionary histories of only a very few polyploids have been reconstructed in detail (e.g. Perný et al., 2005; Lihová et al., 2006, see also Lihová and Marhold, 2006). Several polyploid complexes composed of multiple closely related species have been recognized. The *C. pratensis* complex comprises ancestral diploids from southern Europe and diploid to polyploid species mainly from central and northern Europe (Franzke and Hurka, 2000; Lihová et al., 2003, 2004b). One of the latter is *C. pratensis* s. str. (further referred to as *C. pratensis*), a polymorphic species that displays high cytotype variation due to polyploidy (diploids to heptaploids) and dysploidy (Lökvist, 1956; Mandáková et al., 2013). Diploid and tetraploid subalpine populations from the Alps have been segregated as *C. rivularis* auct. non Schur (e.g. Landolt, 1984; distinct from *C. rivularis* Schur, which is confined to the Southern Carpathians and Bulgarian mountains; see Franzke and Hurka, 2000). *Cardamine amara* is another polyploid complex comprising the widespread diploid *C. amara* subsp. *amara* and several more restricted diploid and tetraploid allopatric or parapatric taxa (Lihová et al., 2004a). In the Alps, the subspecies *amara* co-occurs with its autotetraploid derivative subspecies *austriaca*, although the latter usually grows at higher altitudes (Marhold, 1999).

The members of both polyploid complexes are outcrossing perennials capable of vegetative reproduction (by rhizome fragmentation, stolons or foliar vivipary), growing in mesic to wet habitats, quite often in sympatry or close vicinity, but on the fine scale separated by different habitat preferences (Lihová and Marhold, 2006). Reproductive barriers between the complexes are strong (Lökvist, 1956), with very few hybrids reported (Lihová and Marhold, 2006). Two such hybrids, the triploid *C. × insueta* and hexaploid *C. schulzii*, were discovered at a high-mountain plateau at Urnerboden (Canton of Uri) in the Swiss Alps in the 1970s, and reported as cases of recent, human-influenced hybridization and allopolyploid speciation (Urbanska-Worytkiewicz and Landolt, 1972; Urbanska-Worytkiewicz, 1977a; Urbanska and Landolt, 1998). The triploid *C. × insueta* was inferred to have arisen from crosses between the sympatric diploids *C. rivularis* auct. (genome RR) and *C. amara* subsp. *amara* (genome AA), and was proposed to represent an RRA hybrid. *Cardamine schulzii* (RRRRRAA) was thought to have originated by autopolyploidization of *C. × insueta* (Urbanska-Worytkiewicz, 1977a, 1980). Early molecular studies (Neuffer and Jahncke, 1997; Urbanska et al., 1997) confirmed the hybrid origin of both *C. schulzii* and *C. × insueta*. Their recent formation was inferred based on their occurrence in anthropogenic habitats, which were created at the beginning of the 20th century due to land use changes (Zimmerli, 1986). *Cardamine × insueta* grows mainly in fertilized hay meadows and *C. schulzii* is found in drainage ditches (Urbanska-Worytkiewicz, 1980; Urbanska and Landolt, 1998). Neither hybrid has been found outside of Urnerboden, although another triploid (RAA, without providing a hybrid name) was

recorded from a neighbouring canton (Lej da Champfèr, Oberengadin, Canton of Graubünden; Urbanska-Worytkiewicz and Landolt, 1972), growing in sympatry with its proposed progenitors, the diploid *C. rivularis* auct. and the tetraploid *C. amara* subsp. *austriaca*, but not studied later.

Our recent cytogenetic and molecular study (Mandáková et al., 2013) has provided several lines of evidence that *C. schulzii* did not originate through the scenario previously postulated (Urbanska-Worytkiewicz, 1977a). Instead of an autopolyploid derivative of *C. × insueta*, we have inferred *C. schulzii* to be a trigeneric hybrid containing genomes derived from *C. amara* (A), *C. rivularis* auct. (R) and *C. pratensis* (P). Two cytotypes of *C. schulzii* were found, hypopentaploid (PPRRA) and hypohexaploid (PPPPRA). The presence of *C. pratensis* at the Urnerboden site had already been noted by Urbanska et al. (1997), but regarded as introduced there only very recently (in the 1990s) and not considered to be involved in hybrid formation at all. The allopolyploid genomes of *C. × insueta* and *C. schulzii* have appeared structurally stable (Mandáková et al., 2013), but the frequency of their formation, the amount of genetic variation and evolutionary potential have not been examined in detail so far.

Here we developed and employed progenitor-specific microsatellite markers to closely examine the polyploid origins and ongoing evolutionary processes in the hybridizing *Cardamine* populations in the Swiss Alps. Our aims were: (1) to prove the efficiency of using species-specific microsatellite markers in detecting hybridization events and parental contributions, and use these markers to verify the genome composition of the hybrids reported from the Urnerboden and Lej da Champfèr sites; (2) to test the hypotheses of single versus multiple hybrid formation by analysing the fine-scale genetic structure of the polyploids and their progenitors; and (3) to outline the evolutionary potential of the hybrids for long-term persistence and speciation by assessing their genetic variation and the extent of gene flow within and among the relevant taxa.

MATERIALS AND METHODS

Sampling

Our sampling focused on the populations at Urnerboden (Canton of Uri, Switzerland), delimiting 12 microlocalities there (Fig. 1) but also included comparative material for the parental species from other sites in the Alps and more distant areas (Appendix). Initial identification in the field was based on morphology and later revised by chromosome counting and ploidy level determination. At Urnerboden we identified and sampled *C. amara* subsp. *amara*, *C. rivularis* auct., *C. pratensis* and three hybrid types: triploid *C. × insueta*, hypopentaploids and hypohexaploids. For the hypohexaploids, the name *C. schulzii* (hereafter denoted as *C. × schulzii*; see Discussion for rationale) is fully applicable, since they fit the nomenclatural type and were found at the type locality (microlocality 3). Here we suggest a broad circumscription of *C. × schulzii* that also includes the hypopentaploids (found in neighbouring microlocalities 8 and 10 and having the same parentage; see Results). *Cardamine amara*, *C. rivularis* auct. and *C. × insueta* were abundant across the study area, whereas *C. pratensis* was rare and scattered, and the hypopentaploid and hypohexaploid *C. × schulzii* were

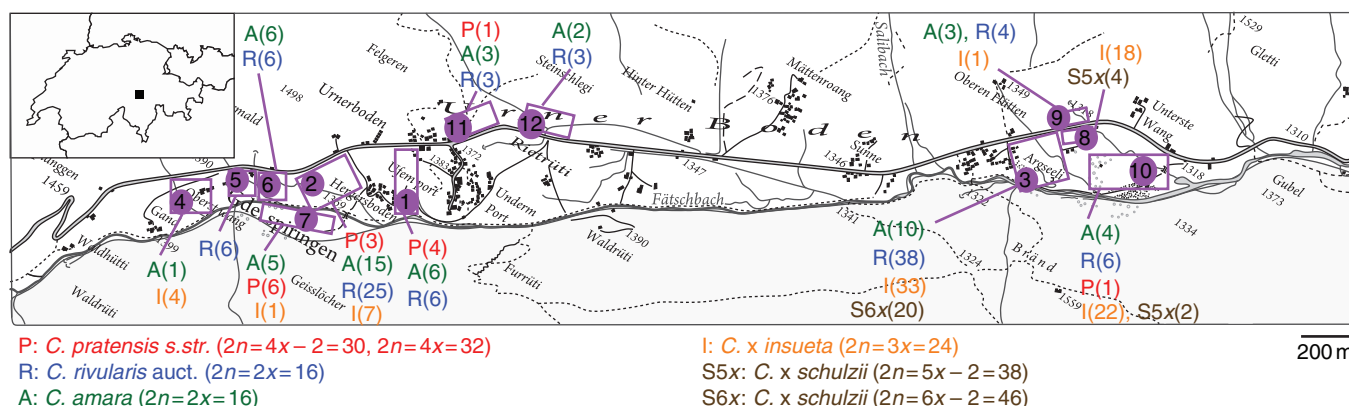


FIG. 1. Map of the Urnerboden valley (Switzerland, Canton of Uri) showing the 12 microlocalities and the numbers of individuals sampled (in brackets).

present only locally. Based on their occurrence and frequency, we sampled 55 individuals of *C. amara*, 96 of *C. rivularis* auct., 15 of *C. pratensis*, 91 of *C. x insueta*, six of hypopentaploid *C. x schulzii* and 22 of hypohexaploid *C. x schulzii* (Fig. 1).

At locations other than Urnerboden, we sampled 12 populations (77 individuals) of *C. amara*, eight populations of *C. rivularis* auct. (63 individuals) and four populations of *C. pratensis* (24 individuals). The hybrids from Lej da Champfär (15 individuals) were also sampled (Appendix).

We collected fresh leaves in the field and dried them in silica gel. Dehydrated leaf tissue was used for both flow cytometry and DNA isolation. Flower buds were collected for chromosome number determination. Representative voucher specimens were deposited in the SAV herbarium (Bratislava, Slovakia). We also analysed herbarium specimens from ZT (ZT35743–35745, ZT35751–35756, Zürich, Switzerland) and OSBU (OSBU6917–6920, Osnabrück, Germany) herbaria that had been collected from the Urnerboden site at the time of hybrid discovery and included in subsequent studies (1970s–1980s), in order to compare their genetic composition and variation with those of the presently found individuals.

Chromosome counting and flow cytometric estimates of ploidy levels

The ploidy level was determined for each individual sampled from the Urnerboden and Lej da Champfär sites and analysed for microsatellite variation. This was achieved by chromosome counting and/or by flow cytometric screening of nuclear DNA content. Mitotic chromosomes were counted from young anthers of flower buds fixed in an ethanol:acetic acid (3:1) mixture and stored in 70 % ethanol at -20°C . Chromosome spreads were prepared following the protocol in Lysak and Mandáková (2013). The chromosomes were stained using 4',6-diamidino-2-phenylindole (DAPI) and observed with an Olympus BX-61 epifluorescence microscope.

Relative nuclear DNA content was measured by flow cytometry using DAPI fluorochrome and following the protocol described in detail in Marhold et al. (2010). To relate the nuclear DNA content to the DNA ploidy levels, plants with known chromosome numbers were measured cytometrically to determine their nuclear DNA content values, and these were used as a reference.

For the other sampled populations, chromosome numbers or ploidy levels were either known from previous literature records or were assessed here by flow cytometry (see Appendix).

Microsatellite marker development

Microsatellite markers were developed from genomic sequence data (generated with the Genome Sequencer FLX system; Roche, Basel, Switzerland) obtained from two individuals sampled at Urnerboden, each representing one of the originally assumed diploid progenitor species, *C. rivularis* auct. and *C. amara*. Our strategy was to target progenitor-specific loci and track them in the hybrids. Species-specific microsatellite loci, expected to amplify in only one of the diploid species and its hybrid derivatives, were identified *in silico* and tested experimentally by PCR.

Sequencing libraries, sequencing of a half-picotiter plate for each sample with the Genome Sequencer FLX, and adapter trimming of the resulting data were performed at the Functional Genomics Center (Zürich, Switzerland). Identification of microsatellite repeats in the unassembled reads and high-throughput primer design were performed using msatcommander 0.8.2 (Faircloth, 2008). To ensure the ability of the microsatellites to discriminate between the two *Cardamine* genomes, the primers designed for each species were compared with the reads of the second species using the stand-alone version of BLAST (Altschul et al., 1990). The results of the BLAST searches were visualized using the PLAN web application (He et al., 2007). Potential species-specific markers were selected and their specificity was verified by cross-amplification tests. More details on marker development, cross-amplifications and marker characteristics, including the primer sequences, are presented in Supplementary Data 'Marker Information' and Table S1.

At the time of marker development, the involvement of *C. pratensis* in the hybrid origin was not expected, and thus screening for loci specific to *C. pratensis* was initially not done. However, due to its close relatedness to *C. rivularis* auct. (also confirmed by our initial PCR tests), *C. rivularis* auct. specificity extends well to *C. pratensis*: the *C. rivularis* auct.-specific loci amplified well also in *C. pratensis*, whereas *C. amara*-specific loci did not. The presence of specific alleles distinguishing between *C. rivularis* auct. and *C. pratensis* were tracked in the hybrids. In total, ten *C. amara*-specific microsatellite loci (hereafter denoted as Cama loci) and six *C. rivularis* auct./*C. pratensis*-specific loci

(denoted as Criv loci) that showed successful amplification and unambiguous allele determination across multiple individuals were employed in the final analyses. In addition, four loci, originally considered as *C. amara*-specific, were found to amplify well also in *C. rivularis* auct. and *C. pratensis* (Supplementary Data 'Marker Information' and Table S1). Since our initial tests indicated that allele size differences may exist among the species, these four shared loci (denoted here as C-all loci) were also employed. Thus, variation at 20 microsatellite loci was finally examined.

Microsatellite amplification and genotyping

Total genomic DNA was isolated using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Microsatellite loci were amplified in five different multiplex reactions, with two to nine loci per reaction. The PCR was conducted in a reaction volume of 10 μ L using a Multiplex PCR Kit (Qiagen). Primer concentrations ranged from 0.05 to 0.4 μ M. Forward primers were fluorescently labelled using 6-FAM, NED, PET and VIC fluorescent dyes. The thermocycling conditions were 95 °C for 15 min, 25–30 cycles of 94 °C for 30 s, 55–58 °C for 90 s and 72 °C for 1 min, followed by the final extension step of 60 °C for 30 min. The PCR products were submitted for fragment analysis (using an ABI 3100 Avant capillary sequencer at the BITCET Consortium, Comenius University, Bratislava) with a GeneScan™ 500 LIZ® (Life Technologies) internal size standard. Allele sizes were recorded using DAX software (Van Mierlo Software Consultancy, The Netherlands). To check the consistency of amplification and allele determination, both within- and among-plate replicates were included.

Data analyses

Three datasets were assembled: (1) ten Cama loci \times 266 individuals of *C. amara* and the hybrids; (2) six Criv loci \times 333 individuals of *C. rivularis* auct., *C. pratensis* and the hybrids; and (3) four C-all loci \times 464 individuals of all three progenitor species and the hybrids.

Due to the allele copy number ambiguity in these datasets, which encompassed both auto- and allopolyploids of different ploidy levels, determination of precise genotypes and allele frequencies was impossible. This limited the statistics and analyses available for data evaluation, although some advances in treating codominant microsatellite data in polyploid species have recently been achieved (discussed in Clark and Jasieniuk, 2011), and were utilized here. Here we employed two approaches to data treatment/coding to describe genetic patterns and compute genetic diversity and distances. The first corresponded to the multilocus genotype data (more accurately termed 'multilocus allele phenotypes', since individual alleles were recorded but with unknown copy numbers in polyploids; Sampson and Byrne, 2012). In the second, we coded each allele as a dominant marker, thus generating a binary presence/absence matrix (e.g. Vallejo-Marin and Lye, 2013). Conversion between these two data formats was done in R 2.15.1 (R Core Team, 2012) using the POLYSAT 1.3–0 package (Clark and Jasieniuk, 2011).

Genetic affinities between the progenitors and hybrids were explored by exact multilocus matches (with the clone assignment

function in POLYSAT) and allele-sharing patterns. To examine genetic structure, both principal coordinates analysis (PCoA) and neighbour-joining clustering (NJ) were done in FAMD 1.25 (Schlüter and Harris, 2006), using two different genetic distance measures. The Bruvo distance (Bruvo et al., 2004) was calculated in POLYSAT from the multilocus allele phenotype data as a measure of genetic distance accounting for mutational distance between the alleles and allowing for allele copy number ambiguity, best suited for autopolyploids (in certain circumstances applicable to allopolyploids; Clark and Jasieniuk, 2011). The other distance was based on Jaccard's similarity coefficient, which does not take shared absence into account, and was calculated in FAMD from the binary presence/absence data matrix. For the Cama loci dataset, the Bruvo distance was an appropriate measure, since no allopolyploids were expected (considering the A subgenomes; see Results). The Criv and C-all loci datasets included individuals that could be regarded as allopolyploid, and because the progenitor species commonly displayed the same or very close alleles, for them the Bruvo distance was not appropriate (Clark and Jasieniuk, 2011), and the Jaccard coefficient was used.

Bayesian model-based clustering was employed, using STRUCTURE 2.3.3 (Pritchard et al., 2000), with an approach that enables the handling of dominant markers and genotype ambiguity in the codominant dataset (Falush et al., 2007). Due to the multiple ploidy levels present in our datasets, however, we opted for the dominant, presence/absence coding. For the hybrid individuals, only unique genotypes were kept in the datasets. This analysis was run to identify homogeneous genetic clusters and detect genetic admixture. Five to ten replicates were run for each $K = 1–10$ (user-defined number of clusters), with a burn-in length of 100 000 generations and data collection for an additional 1 000 000 generations, using the admixture model and uncorrelated allele frequencies. The analyses were performed on the Bioportal of the University of Oslo (www.bioportal.uio.no; Kumar et al., 2009). The STRUCTURE output data were parsed using STRUCTURE HARVESTER (Earl and vonHoldt, 2012) to determine the optimal K value, following the method of Evanno et al. (2005). Alignment of cluster assignments across replicate analyses was then conducted in CLUMPP 1.1.2b (Jakobsson and Rosenberg, 2007) and visualized using DISTRUCT 1.1 (Rosenberg, 2004).

The genetic diversity of each population/taxon was expressed as the total number of alleles observed over all loci (A), the average number of alleles per locus (A'), the average number of alleles per locus per individual (A_i'), the proportion of (partial) heterozygous individuals (H_o), the percentage of polymorphic loci [$P(\%)$], the number of different multilocus allele phenotypes (N_{phe}) and the Shannon diversity index (a statistic based on genotype frequencies), computed manually or in POLYSAT.

RESULTS

Chromosome numbers and ploidy levels

Chromosome counting and flow cytometry showed that all *C. rivularis* auct. individuals sampled from Unerboden were diploid ($2n = 2x = 16$). *Cardamine pratensis* was tetraploid ($2n = 4x = 32$) or hypotetraploid ($2n = 4x - 2 = 30$) at

Urnerboden, the sole exception being a single hypopentaploid individual (determined as $2n = 3x = 38$; however, this individual turned out to be a *C. rivularis* auct. \times *C. pratensis* hybrid; see below). From the other localities, *C. rivularis* auct. included diploids and tetraploids, whereas *C. pratensis* was (hypo)tetraploid and (hypo)hexaploid. *Cardamine amara* from Urnerboden was diploid (a few tetraploids were also found but not analysed here), and from the other localities both diploid (i.e. subsp. *amara*, $2n = 2x = 16$) and tetraploid populations (subsp. *austriaca*, $2n = 4x = 32$) were recorded (Appendix).

Three ploidy levels were found among the hybrid individuals: most frequently triploids ($2n = 3x = 24$, *C. \times insueta* from Urnerboden, hybrids from Lej da Champfèr), less frequently hypohexaploids ($2n = 6x - 2 = 46$, *C. \times schulzii*, Urnerboden microlocality 3), and very few hypopentaploids ($2n = 5x - 2 = 38$, *C. \times schulzii*, Urnerboden microlocalities 8 and 10). All the ploidy levels determined here or known from the literature are summarized in the Appendix.

Microsatellite data diversity, multilocus matches and patterns of allele sharing

Allelic variation. The dataset of the ten Cama loci encompassed 102 alleles in total (3–22 alleles/locus), that of the six Criv loci encompassed 74 alleles (6–21/locus) and that of the four shared C-all loci encompassed 49 alleles (8–18/locus). Three Criv loci (Criv15, Criv18 and Criv23) were found to have been duplicated, but were kept in the final analyses due to the scarcity of *C. rivularis* auct./*C. pratensis*-specific loci revealed during marker development (Supplementary Data ‘Marker Information’). All progenitor-specific loci amplified well in the hybrids from Urnerboden. Only one Criv locus (Criv34) was not amplified in the triploids from Lej da Champfèr, but this locus amplified in only a few individuals of

the sympatric *C. rivularis* auct. Samples of *C. rivularis* auct. and *C. pratensis* from Urnerboden shared alleles at each of the Criv and C-all loci, but we also identified *C. pratensis*-specific (i.e. not present in *C. rivularis* auct.; 34 alleles) and *C. rivularis* auct.-specific alleles (eight alleles) (Table 1).

Multilocus matches and allele sharing at Cama loci. Multilocus matches at Cama loci were found for several individuals of *C. amara* within the same population. No exact match was observed between *C. amara* and hybrid individuals. Nevertheless, allele-sharing patterns revealed that each Cama allele observed in the Urnerboden hybrids was also found in the sympatric *C. amara* (Supplementary Data Table S3). The patterns at the Cama loci in the Lej da Champfèr triploid hybrid were more complex; several alleles present in the hybrid were not found in the sympatric *C. amara*, but only in some other populations, and two hybrid-specific alleles, not found elsewhere, were detected.

Multilocus matches and allele sharing at Criv loci. At the Criv loci, multilocus matches were observed within several populations of the progenitors, and also between *C. \times insueta* and sympatric *C. rivularis* auct. Four exact matches between this triploid and its progenitor were detected, with 28 of 91 sampled *C. \times insueta* individuals showing matches with one to five individuals of *C. rivularis* auct. The matches were not only among individuals from the same microlocality or very close microlocalities (e.g. 3, 8 and 10), but also between those from microlocalities spaced farther apart (e.g. 1 and 3). The rest of the *C. \times insueta* individuals – those not matching the genotypes *C. rivularis* auct. exactly – possessed only the alleles found in sympatric *C. rivularis* auct. The Criv alleles recorded in *C. \times schulzii* were also observed in sympatric *C. rivularis* auct. and/or *C. pratensis*, except for one allele in the hypohexaploids, and two in the hypopentaploids that were not found elsewhere. *Cardamine rivularis* auct.-specific (not present in *C. pratensis*)

TABLE 1. Patterns of allele sharing between *Cardamine pratensis* (P) and *C. rivularis* auct. (R) individuals from the Urnerboden locality, and occurrence of *C. pratensis*-specific and *C. rivularis*-specific alleles in the hybrids

Locus	Number of alleles								
	Total	Present in P/R group ¹	Shared by P and R ²	Specific to P ³	Specific to R ⁴	Specific to P ³ , present in <i>C. insueta</i> ⁵	Specific to P ³ , present in 5x/6x <i>C. schulzii</i>	Specific to R ⁴ , present in <i>C. insueta</i>	Specific to R ⁴ , present in 5x/6x <i>C. schulzii</i>
Cama7 (C-all)	12	11	1	4	0	0	0/0	0	0/0
Cama19 (C-all)	8	8	2	3	2	0	0/0	0	0/0
Cama23 (C-all)	18	13	3	3	3	0	0/1	0	0/0
Cama25 (C-all)	11	10	5	2	1	0	0/1	0	0/0
Criv2	8	8	2	4	0	0	1/0	0	0/0
Criv15	7	7	4	2	0	0	1/1	0	0/0
Criv16	16	16	2	6	1	0	0/0	0	0/0
Criv17	13	13	1	2	0	0	0/1	0	0/0
Criv18	6	6	3	1	0	0	0/0	0	0/0
Criv23	21	21	3	6	1	0	0/3	1	1/0
Criv34	11	11	2	1	0	0	1/0	0	0/0
Total	131	124	28	34	8	0	3/7	1	1/0

¹ Alleles present in *C. pratensis* and *C. rivularis* auct. within and outside Urnerboden populations.

² Alleles shared between the two taxa in Urnerboden, irrespective of their presence or absence outside Urnerboden.

³ Alleles present in *C. pratensis* from Urnerboden and absent from *C. rivularis* auct. from Urnerboden, irrespective of their presence or absence outside Urnerboden.

⁴ Alleles present in *C. rivularis* auct. from Urnerboden and absent from *C. pratensis* from Urnerboden, irrespective of their presence or absence outside Urnerboden.

alleles were rare, and one such allele was recorded in both *C. × insueta* and hypopentaploid *C. × schulzii* (Table 1). *Cardamine pratensis*-specific (not present in *C. rivularis* auct.) alleles, on the other hand, were more abundant and were found also in *C. × schulzii*, with seven such alleles in the hypohexaploids and three in the hypopentaploids, but not in *C. × insueta* (Table 1; see also Supplementary Data Table S3). These numbers refer only to the *C. rivularis* auct. and *C. pratensis* samples from Urnerboden, due to the taxonomic uncertainty of the other sampled populations (see below). The triploid hybrid from Lej da Champfèr did not show an exact match with its progenitor, but all of its alleles were observed in sympatric *C. rivularis* auct.

Allele sharing at C-all loci. At the C-all loci, all alleles found in the Urnerboden hybrids were detected in the sympatric parental species. This, with the exception of one allele, also holds true for the Lej da Champfèr triploid hybrid. Allele-sharing patterns at the C-all loci clearly showed additivity for all four different types of hybrid (Table 2; Supplementary Data ‘Marker Information’ and Table S3). Even though allele sharing between the progenitors was common, progenitor-specific alleles could also be found and identified in the hybrids. *Cardamine amara*-specific and *C. rivularis* auct./*C. pratensis*-specific alleles were detected in *C. × insueta*, *C. × schulzii* and the Lej da Champfèr triploid. In addition, two *C. pratensis*-specific alleles were confirmed in the hypohexaploid *C. × schulzii* (Table 2).

Numbers of alleles in hybrids, indicating numbers of parental genomes. The numbers of alleles per locus and individual (excluding the duplicated loci) corresponded to the ploidy levels of the progenitor species and suggested the numbers of respective genomes in the hybrids to be one A (*C. amara*-derived) genome in *C. × insueta* and *C. × schulzii*, two R (*C. rivularis* auct.-derived) genomes in *C. × insueta* and two A genomes and one R genome in the triploid from Lej da Champfèr. The numbers of Criv alleles per locus in *C. × schulzii* individuals were lower than the expected number of P + R genomes (four and five in the hypopentaploids and hypohexaploids, respectively): a maximum of three and two alleles were observed in the hypopentaploids and hypohexaploids, respectively.

Genetic diversity of hybrids. Unequal sample sizes hampered statistical comparison of diversity parameters among populations

and taxa, but some patterns are obvious. Hybrids harboured substantially less genetic variation than the progenitors (Supplementary Data Table S2). *Cardamine × schulzii*, including both cytotypes, was invariable at the Cama loci; at the Criv and C-all loci we found two multilocus allele phenotypes, with one displayed by all the hypohexaploids and the other by all the hypopentaploids. *Cardamine × insueta* possessed more genetic variation, with three multilocus allele phenotypes at the Cama loci, 16 at the Criv loci and six at the C-all loci (N_{phe} in Supplementary Data Table S2), but all diversity parameters were still below those of the parental species. The triploid from Lej da Champfèr showed two multilocus allele phenotypes at the Cama loci, two at the Criv loci and one at the C-all loci.

The microsatellite data retrieved from ZT herbarium specimens were, due to low DNA quality, only fragmentary. Nevertheless, all the alleles found in these old specimens were found also in the present-day populations at Urnerboden. In the OSBU herbarium specimens all loci could be amplified, and the multilocus phenotypes that were obtained matched the currently sampled individuals of *C. × insueta* and the hypohexaploid *C. × schulzii*.

Genetic structure at Cardamine amara-specific (Cama) loci

Both the PCoA plot (Fig. 2A) and the NJ tree (Supplementary Data Fig. S1) manifested shallow structure among the analysed accessions. The populations outside the Alps, however, appeared slightly differentiated from the Alpine ones (Urnerboden and two other Swiss populations). Extensive variation was found among the *C. amara* individuals from Urnerboden compared with the other sampled populations, but with no clustering related to the microlocalities. All Urnerboden hybrid individuals displayed only three multilocus allele phenotypes, which were placed close to the Alpine *C. amara*. One of these was shared by most *C. × insueta* (83/91 individuals from all microlocalities) and all of the *C. × schulzii* individuals, while the other two phenotypes characterized eight individuals of *C. × insueta* from microlocalities 3 and 8. The Lej da Champfèr triploid appeared in a somewhat distant position.

TABLE 2. Patterns of allele sharing and specificity at C-all loci

Locus	Total	Number of alleles						
		Present/specific P/R group ¹	Present/specific A	Shared by P/R and A	Present in hybrids, specific to P/R group ²	Present in hybrids, specific to A ³	Present in hybrids, equivocal origin ⁴	Present in <i>C. schulzii</i> , specific to P ⁵
Cama7 (C-all)	12	11/7	5/1	4	0	0	2	0
Cama19 (C-all)	8	8/3	5/0	5	0	0	1	0
Cama23 (C-all)	18	13/12	6/5	1	3	2	1	1
Cama25 (C-all)	11	10/5	6/1	5	5	1	1	1
Total	49	42/27	22/7	12	8	3	5	2

¹ Alleles present in/specific for *C. pratensis* and *C. rivularis* auct. within and outside Urnerboden populations.
² Alleles unequivocally originating from *C. pratensis* or *C. rivularis* auct.
³ Alleles unequivocally originating from *C. amara*.
⁴ Alleles not discriminating between the *C. pratensis*/*C. rivularis* auct. and *C. amara*.
⁵ Alleles present in hypohexaploid *C. × schulzii* and unequivocally originating from *C. pratensis* from Urnerboden (none such were found in hypopentaploid *C. × schulzii*).
P, *Cardamine pratensis*, R, *C. rivularis* auct., A, *C. amara*.

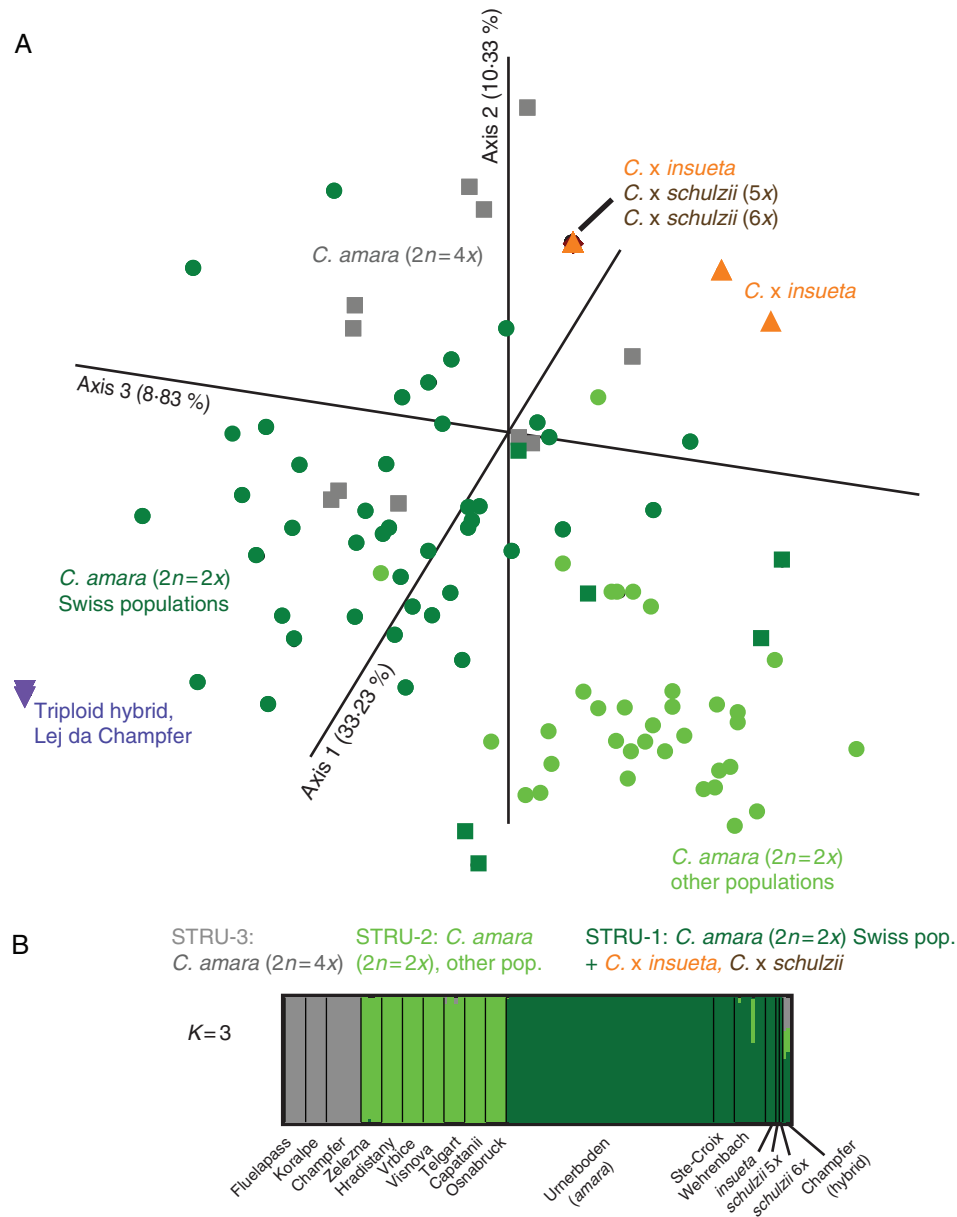


FIG. 2. Genetic structure of 266 individuals based on ten *Cardamine amara*-specific (Cama) microsatellite loci. (A) Principal coordinates analysis based on the Bruvo coefficient. Percentage of variation represented by the axes is indicated. Colours and symbols indicate species/group affiliation (dark green circles stand for the *C. amara* accessions from Urnerboden, dark green squares for those from the other two Swiss populations). (B) Bayesian model-based clustering at $K = 3$. Vertical lines demarcate the populations, with the widths of the corresponding boxes proportional to the numbers of individuals analysed in the populations. The colouring indicates each individual's proportional cluster assignment. Population codes are presented in the Appendix.

Bayesian model-based clustering with optimal value $K = 3$ revealed the following genetic clusters (Fig. 2B): STRU-1, diploid *C. amara* from Urnerboden and the other Swiss localities, as well as all Urnerboden hybrids; STRU-2, diploid *C. amara* from the other studied localities; and STRU-3, tetraploid *C. amara*. Equivocal assignment was obtained for the Lej da Champfer triploid hybrid.

Genetic structure at *Cardamine rivularis* auct./*C. pratensis*-specific (*Criv*) loci

The NJ tree displayed three clearly divergent clusters and four isolated branches in intermediate positions (Fig. 3A). The three

main clusters corresponded to: (1) *C. rivularis* auct. from Urnerboden, including all *C. x insueta* individuals; (2) *C. pratensis* from Urnerboden and the other sampled populations, along with *C. rivularis* auct. (diploids and tetraploids) from the Austrian localities; and (3) diploid *C. rivularis* auct. from the Swiss populations (other than Urnerboden), including also the Lej da Champfer triploid hybrid. Grouping of individuals within the *C. rivularis* auct. + *C. x insueta* cluster did not follow the different Urnerboden microlocalities. Finally, the four intermediate branches consisted of hypohexaploid *C. x schulzii* (one branch), hypopentaploid *C. x schulzii* (one branch), and three *C. pratensis* individuals (two branches, including the individual with 38 chromosomes). These clustering

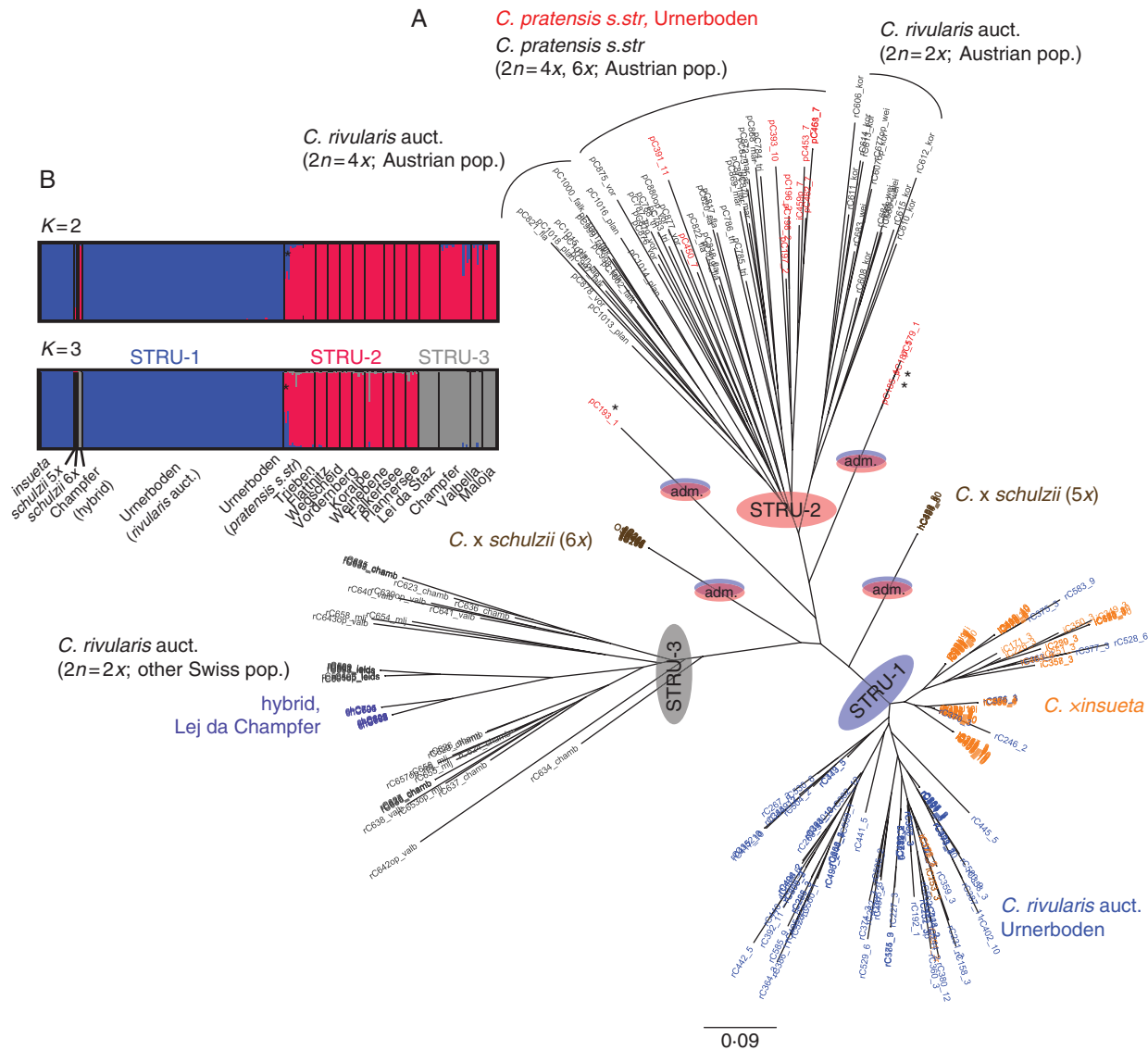


FIG. 3. Clustering of 333 individuals based on six *Cardamine rivularis auct./C. pratensis*-specific (Criv) microsatellite loci. (A) Neighbour-joining tree based on the Jaccard coefficient. Colours indicate species/group affiliation. (B) Bayesian model-based clustering at K=2 and K=3. For explanation of graph see legend of Fig. 2. Asterisks highlight three individuals of *C. pratensis* from Urnerboden showing genetic admixture between the STRU-1 and STRU-2 clusters.

patterns indicate the distinct origins of the four types of hybrid, as well as the intermediacy (additivity) of the hypohexaploid and hypopentaploid *C. x schulzii* containing both R and P genomes. These results also indicate genetic distinction between *C. pratensis* and *C. rivularis auct.* at Urnerboden, whereas the patterns of variation among the samples from other sites would argue against distinguishing between these two species as currently circumscribed. Indeed, our results revealed several genetic entities related to geography and ploidy levels, potentially representing cryptic species, which need to be explored and delimited in future (being beyond the scope of the present paper).

Bayesian model-based clustering suggested two consistent clustering solutions, K=2 (highest ΔK value) and K=3 (the second highest ΔK , also reaching the plateau of the likelihood distribution of K). At K=2, *C. rivularis auct.* from Urnerboden and *C. x insueta* (NJ cluster 1 above) were separated from the rest of

the individuals, whereas at K=3 the three clusters corresponded to those in the NJ tree (Fig. 3B). In both cases, *C. x schulzii* (both cytotypes, but mainly hypohexaploid) and the three individuals of *C. pratensis* from two intermediate branches (marked with asterisks in Fig. 3) showed admixed ancestry drawn from two clusters containing Urnerboden individuals of *C. pratensis* and *C. rivularis auct.*, respectively.

The PCoA plot was congruent with the results of the above analyses, supporting the three main groupings and the intermediacy of *C. x schulzii* (Supplementary Data Fig. S2).

Genetic structure at shared (C-all) loci

The NJ tree recovered several mostly smaller clusters, which could be placed in three main groupings (Supplementary Data Fig. S3), as also recovered by PCoA (Fig. 4A). *Cardamine amara* was clearly separated from both *C. rivularis auct.* and

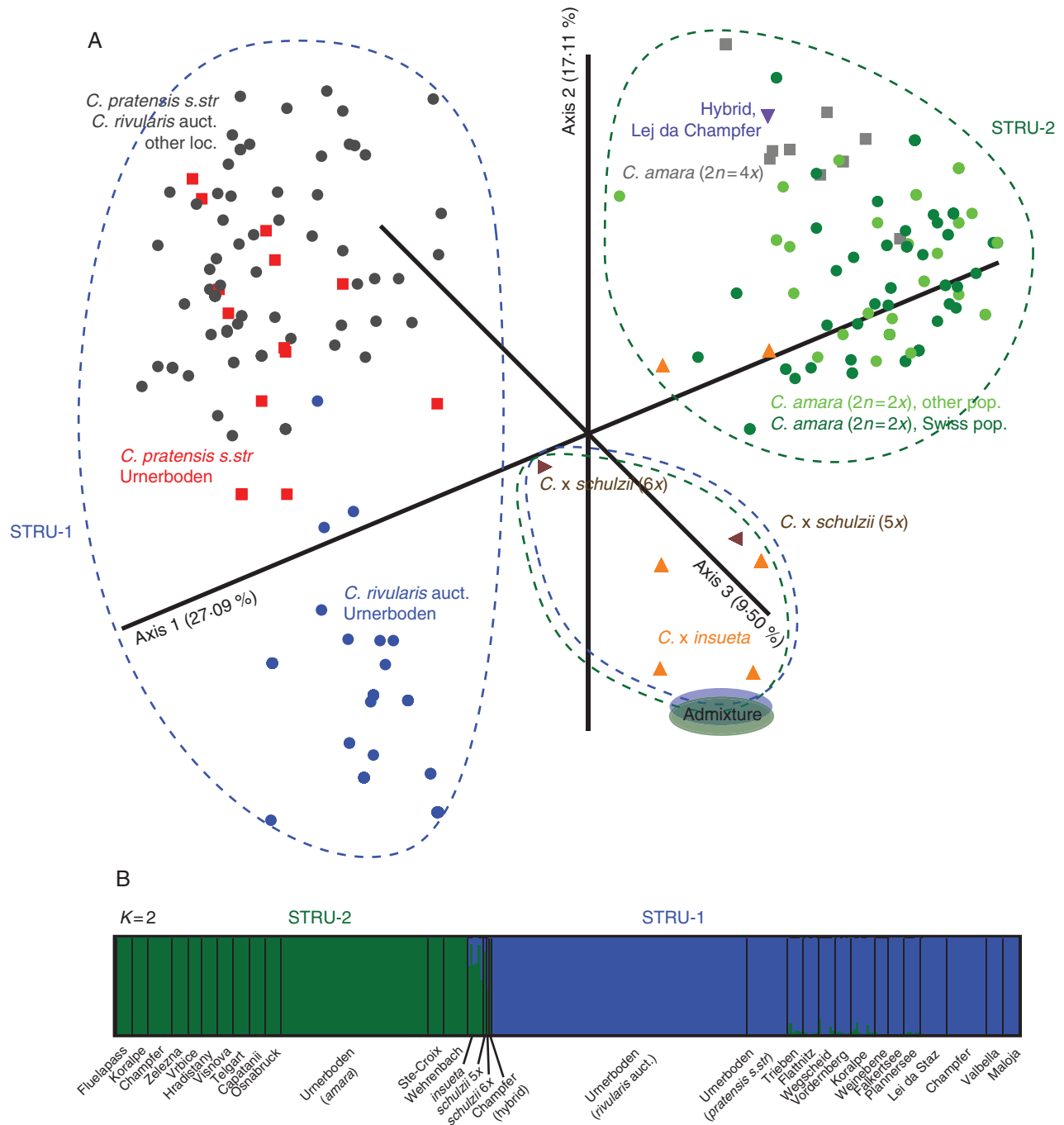


FIG. 4. Genetic structure of 464 individuals based on four shared (C-all) microsatellite loci. (A) Principal coordinates analysis based on the Jaccard coefficient. Percentage of variation represented by the axes is indicated. Colours and symbols indicate species/group affiliation. (B) Bayesian model-based clustering at $K = 2$. For explanation of the graph see legend of Fig. 2.

C. pratensis along the first axis (Fig. 4A). The second axis, in turn, showed the distinctiveness of *C. rivularis* auct. from Uernerboden. The position of most Uernerboden hybrid individuals – hypohexaploid and hypopentaploid *C. × schulzii* and most *C. × insueta* (83 individuals representing four multilocus phenotypes) – was clearly intermediate. Eight individuals (from microlocalities 3 and 8) of *C. × insueta*, displaying two multilocus phenotypes, were at the variation margin of

C. amara (Fig. 4A), although the NJ tree resolved them as a separate cluster in an intermediate position (Supplementary Data Fig. S3). The Lej da Champfer triploid hybrid appeared close to tetraploid *C. amara*.

Bayesian model-based clustering at optimal $K = 2$ differentiated between *C. amara* in one group and both *C. rivularis* auct. and *C. pratensis* in the other, with admixed ancestry detected in all three Uernerboden hybrids (Fig. 4B).

DISCUSSION

Newly arisen allopolyploid species provide an excellent resource for studying the processes of polyploid speciation and evolution at early stages. *Cardamine* × *schulzii*, discovered at a single site at Urnerboden, Switzerland, was one of the classic examples of neoallopolyploidy documented by cytological, experimental breeding and early molecular studies (summarized by Urbanska *et al.*, 1997). Since that time, however, powerful cytogenetic and molecular methods that can provide deeper insights into reticulate and polyploid evolution have become available (Hegarty and Hiscock, 2005). Indeed, the multidisciplinary approach applied recently (Mandáková *et al.*, 2013) rejected the postulated autoallopolyploid origin of *C.* × *schulzii* and showed that it is a trigonomic hybrid. Despite the close relatedness of the *C. rivularis* auct. and *C. pratensis* genomes, a few *C. pratensis*-specific genomic features proved the contribution of this species and made it possible to determine the ratio of P to R chromosomes and to infer the genome composition of the hypopentaploids as PPRRA and the hypoheptaploids as PPPPRA (Mandáková *et al.*, 2013). Here we follow these recent findings and, using high-resolution multilocus genotyping with species-specific markers, we provide additional evidence for the inferred hybridization scenarios and also gain insight into the genetic structure and variation of the parental species and their hybrid progeny.

Progenitor-specific microsatellite loci and alleles verify the hybrid origins and genome compositions of the allopolyploid Cardamine species at Urnerboden

All progenitor-specific microsatellite loci (Cama, Criv loci) discriminating between *C. amara* and *C. pratensis/C. rivularis* auct. amplified well in both *C. × schulzii* and *C. × insueta*, confirming their hybrid origins. Additionally, shared C-all loci displayed some species-specific alleles that showed additivity in the studied hybrids. The allelic variation also unequivocally indicates the involvement of *C. pratensis* in the origin of *C. × schulzii*, which supports the recent cytogenomic findings (Mandáková *et al.*, 2013). Several loci present in both *C. rivularis* auct. and *C. pratensis* (Criv and C-all loci) displayed *C. pratensis*-specific alleles, and some of these were found in hypopentaploid and hypoheptaploid *C. × schulzii*, but not in the triploid *C. × insueta*. Also, the ordination (PCoA) and clustering (NJ) methods congruently showed the intermediacy of *C. × schulzii* at Criv loci, strongly supported by the Bayesian model-based clustering that revealed genetic admixture within *C. × schulzii*, reflecting its ancestry in two genetic clusters, one comprising *C. pratensis* and the other *C. rivularis* auct. (both from Urnerboden).

Our finding of only one allele consistently recorded at each *C. amara*-specific (Cama) locus in all Urnerboden hybrid individuals supports the presence of only one A genome. Two R genomes were inferred for *C. × insueta*, in accordance with the expected RRA composition of this triploid. For *C. × schulzii*, however, due to allele sharing by *C. pratensis* and *C. rivularis* auct. and allele copy number ambiguity, the exact number of P and R genomes could not be inferred from the microsatellite data.

Importantly, analyses of specimens collected more than 20 – 30 years ago at Urnerboden (deposited in the ZT and OSBU

herbaria) did not reveal genotypes or alleles distinct from those found in the currently sampled hybrid plants, and also proved the presence of *C. pratensis*-specific alleles in *C. × schulzii*. These findings, in accord with those by Mandáková *et al.* (2013), support our conclusion that the hybrids which we collected are the same as those reported and studied by Urbanska and co-authors.

Multilocus genotyping, which provides a high number of polymorphic and independent markers, has been commonly used to detect hybrid offspring and study hybrid zones. Nevertheless, considerable genotyping effort involving many loci may be needed, especially if divergence between parental species is low and/or later-generation and backcrossed individuals are also present (Vähä and Primmer, 2006). Species-specific markers can significantly facilitate such efforts. The present study illustrates the efficiency of using progenitor-specific microsatellite markers to detect hybridization as well as to infer parental contributions in hybrid progeny. A few other studies have employed a similar approach, for instance to examine interspecific gene flow in poplars (Khasa *et al.*, 2005), recurrent formation in *Tragopogon* polyploids and gene flow among the independent polyploid lineages (Symonds *et al.*, 2010), or to discriminate between two salmon species and recognize their hybrids (Perrier *et al.*, 2011).

Scenarios proposed for the formation of Cardamine × schulzii

The exclusion of the autoallopolyploid origin of *C. × schulzii* from the triploid *C. × insueta* raised the question of the actual parentage of the hypoheptaploid and hypopentaploid hybrids. The inferred genome composition, cpDNA haplotype sharing (implying that R genomes were maternally inherited) and gametes (polarized, unreduced and aneuploid) observed in *C. × insueta* suggested that the most plausible route for the formation of the hypoheptaploid *C. × schulzii* (RPPPPA) is via hybridization between *C. × insueta* (assuming an RA egg cell with 16 chromosomes) and *C. pratensis* (unreduced PPPP pollen with 30 chromosomes) (Mandáková *et al.*, 2013). Similarly, the hypopentaploid *C. × schulzii* (RRPPA) has probably originated via hybridization between *C. × insueta* (unreduced RRA egg cell) and *C. pratensis* (reduced PP pollen), one of the gametes being likely to be aneuploid ($n - 1$; Mandáková *et al.*, 2013). The origin of both cytotypes of *C. × schulzii* via *C. × insueta* is strongly favoured by the microsatellite data, considering the fact that the same multilocus allelic phenotype at Cama loci was shared by all *C. × schulzii* and most *C. × insueta* individuals. In an alternative scenario, a *C. pratensis* × *C. rivularis* auct. hybrid might have arisen first, and then hybridized with *C. amara*. Such a hypopentaploid hybrid plant (*C. pratensis* × *C. rivularis* auct.) with 38 chromosomes has indeed been identified by the present microsatellite data. Genetic admixture was also indicated in two 30-chromosome plants of *C. pratensis*, which probably represent such hybrids as well. Nevertheless, the latter scenario of the origin of *C. × schulzii* is less likely because its multilocus allelic phenotypes at Criv loci differ from those of the *C. pratensis* × *C. rivularis* auct. hybrids, and also the abundance of *C. × insueta* contrasts with the scarcity of *C. pratensis* × *C. rivularis* auct. hybrids.

The observed allelic variation in both *C. × insueta* and *C. × schulzii* supports the inference of their recent origins, as

originally proposed by Zimmerli (1986) on the basis of their occurrence in habitats created after land-use changes in the early 20th century. The level of allele sharing between the progenitors and the allopolyploids, with very few non-parental alleles detected in *C. × schulzii*, only at Criv loci, favours their recent origins. These seemingly *C. × schulzii*-specific alleles might represent alleles present in the progenitors but not sampled here (the sampling of *C. rivularis* auct. was extensive, but still some alleles may have escaped, whereas that of *C. pratensis* was indeed limited due to the species scarcity at the locality) or they may have evolved in the allopolyploid genomes. Human-induced habitat disturbance and generation of new niches have apparently played a significant role in the hybrid formation and establishment in Urnerboden (Urbanska and Landolt, 1998), as has been recognized also in other plant groups (Levin et al., 1996; see also Bleeker and Hurka, 2001; Lihová et al., 2007).

Multiple origins and interbreeding explain genetic variation in triploid Cardamine × insueta

It is now widely recognized that multiple and recurrent formations are common in allopolyploids (Soltis and Soltis, 1999). Recurrent hybridization and polyploidization events increase genetic variation and result in distinct hybrid genotypes, which can create additional diversity by interbreeding, differential homeologous recombination and segregation. Such processes can yield a highly variable polyploid species or even multiple cryptic species (Soltis and Soltis, 1999; Shimizu-Inatsugi et al., 2009; Symonds et al., 2010). In our study, both diploid progenitors displayed substantial variation at the studied microsatellite loci (despite their ability to reproduce clonally), enhancing the likelihood of detecting independent origins arising from genetically distinct parental individuals. Nevertheless, neither *C. amara* nor *C. rivularis* auct. showed geographically correlated genetic structure within the Urnerboden valley. Apparently, intraspecific gene flow is efficient within these outcrossing species, hindering distinction between multiple (in the sense of polytopic) and single origins of the studied hybrids. Moreover, even though discerning multiple hybrid origins is easier in recent polyploids, inferring the exact number of origins may be hampered by recombination and segregation of homeologous chromosomes, gene flow among the polyploids and backcrosses accumulating in later generations (Soltis and Soltis, 2009) – processes that may have obscured the true number of independent origins in this system.

Three multilocus genotypes were revealed in *C. × insueta* at Cama loci, as many as 16 at Criv loci, and six at C-all loci. Two of the three Cama genotypes in *C. × insueta*, however, are very similar to each other, differing at a single locus, which might be due to a recent mutation in the hybrid. Thus, only two of these multilocus genotypes clearly reflect distinct parental origins. Since *C. × insueta* contains only one A genome, these two genotypes observed at Cama loci could suggest at least two independent origins, i.e. involvement of two different male gametes. At Criv loci, much higher variation was found in *C. × insueta*, but recombination and segregation probably generated some of this variation. Although sexual reproduction is apparently limited in *C. × insueta* – as evidenced by meiotic irregularities, the prevalence of male-sterile plants with non-dehiscent anthers

(Urbanska-Worytkiewicz, 1977b; Mandáková et al., 2013) and efficient vegetative propagation (J. Zozomová-Lihová et al., pers. obs.; Urbanska-Worytkiewicz, 1980) – viable gametes could be produced rarely and engage in sexual reproduction. Polarized meiosis (characterized by asymmetrical chromosome segregation), giving rise to two reduced euploid gamete types, RA and R, in addition to the unreduced RRA type, was previously reported and supported by experimental cross-pollinations (Urbanska-Worytkiewicz, 1977b). The four exact multilocus matches between *C. × insueta* and *C. rivularis* auct. could be regarded as evidence of independent origins, but some of these matches could also be due to recombination in the diploids or triploids (given the rather low number of investigated Criv loci and the high frequencies of most alleles). Thus, although we cannot infer the exact number of hybridization events that have resulted in *C. × insueta*, the relatively large genetic variation of *C. × insueta* is likely due to both multiple origins and interbreeding. Whether and to what extent backcrossing with *C. rivularis* auct. has shaped this variation remains unknown. Nevertheless, experimental backcrossing by Urbanska-Worytkiewicz (1977b) did yield diploid to pentaploid offspring, and although we did not detect individuals such as RRRA and RRRRA (raised experimentally by Urbanska-Worytkiewicz) in our study, some RR (*C. rivularis* auct.) and RRA (*C. × insueta*) individuals might be products of backcrossing.

Two hybridization events gave rise to two distinct cytotypes of Cardamine × schulzii

The present microsatellite data support two distinct hybridization events between *C. × insueta* and *C. pratensis*, giving rise to two cytotypes of *C. × schulzii*. These cytotypes were clearly genetically distinct, as seen from the alleles at Criv and C-all loci, and found in neighbouring microlocalities. Given the genetic uniformity within these two cytotypes, however, we can infer that each cytotype arose only once. Interestingly, the hypopentaploids have never previously been reported, meaning that they either escaped sampling or arose subsequently. The allelic composition of the old herbarium specimens matched either *C. × insueta* or hypohexaploid *C. × schulzii*.

Occurrence of another triploid hybrid with similar parentage but reciprocal genomic composition

We documented another hybridization event, from Lej da Champfèr, in accord with the earlier report by Urbanska-Worytkiewicz and Landolt (1972). We confirmed the genomic composition of this triploid, originally inferred as RAA, based on the number of progenitor-specific alleles retrieved from the triploid individuals. This triploid resembles *C. × insueta* morphologically (Urbanska-Worytkiewicz and Landolt, 1972), but is genetically distinct (as are its co-occurring progenitors from those at Urnerboden). It displayed a total of four different genotypes. This genetic diversity is somewhat surprising, given that it forms a single dense patch, for which a single origin and genetic uniformity would be expected. In addition, at Cama loci the hybrid contained several non-parental alleles, or alleles absent from the sympatric *C. amara*. Possibly these alleles are actually present in sympatric *C. amara* but were not sampled, or the population size and genetic variation of *C. amara* declined since the

hybrid origin. The latter explanation is favoured by the genetic depauperation observed in *C. amara* and by its restricted occurrence on a disturbed lakeshore.

Reproductive behaviour and evolutionary implications of Cardamine allopolyploids

Diverse evolutionary fates of hybridization events, whether or not coupled with polyploidization, can be observed in natural populations. The progeny have a high chance of rapidly vanishing, due to minority cytotype disadvantage and small effective population size as well as likely increased sterility, lower fitness and the absence of suitable available niches. Even if some life history traits and reproductive strategies, such as the perennial habit, vegetative reproduction and self-compatibility, facilitate hybrid establishment, their populations remain genetically depauperate and thus vulnerable to extinction by environmental disturbances (reviewed by Arrigo and Barker, 2012). It has been suggested that recent polyploid species have higher extinction rates than their diploid relatives, and thus those that are extant represent rather rare successes (Arrigo and Barker, 2012). Indeed, recent allopolyploid lineage extinction has been observed in *Senecio cambrensis* (Abbott and Lowe, 2004).

Our findings on *C. × schulzii*, gathered several decades after its discovery, disagree to some extent with earlier observations. Previous studies, including field observations, experimental cultivation (Urbanska-Worytkiewicz, 1980) and analyses of pollen fertility (Urbanska et al., 1997), suggested that *C. × schulzii* had a balanced system of sexual and clonal reproduction. The genetic uniformity that we found in both cytotypes of *C. × schulzii*, however, indicates the presence of only vegetative propagation. This agrees with meiotic irregularities and pollen grains of different size and low fertility observed in these plants (Mandáková et al., 2013). Moreover, the peculiar trigonomic composition and the ratio of R/P to A genomes of 4:1 or 5:1 do not favour efficient sexual reproduction either.

Cardamine × schulzii is currently restricted to three adjacent microlocalities (3, 8 and 10), within or close to the locus classicus (locality 3; E. Landolt, ETH, Zurich, pers. comm.), and apparently is not spreading significantly. Thus, *C. × schulzii* remains a rare and locally persisting hybrid derivative without significant evolutionary potential, confined to a particular man-made niche, and is thus highly vulnerable to extinction. We refrain from treating it as a stabilized species as originally described (*C. schulzii*), and instead refer to these hybrid plants as *C. × schulzii*, as has been done in this paper. Sterile hybrids, however, can recover fertility or become widespread even if genetically depauperate (Ainouche et al., 2004; Vallejo-Marin, 2012; Vallejo-Marin and Lye, 2013), and long-term monitoring of *C. × schulzii* is therefore advisable.

The formation of hybrid swarms, another possible outcome of hybridization, depends upon weak reproductive barriers among the progenitors and hybrid derivatives, as well as sufficient gene flow between ploidy levels (e.g. Lihová et al., 2007). At Unerboden, however, barriers to interspecific and interploidal gene flow appear to be rather strong, maintaining the integrity of the progenitor species.

The third possible, and evolutionarily most significant, outcome of hybridization is the formation of a new homoploid

or allopolyploid species. Such a species is usually reproductively isolated from its progenitors, colonizes new habitats or further evolves by genome rearrangements and genetic, epigenetic and transcriptional changes (Hegarty and Hiscock, 2008; Arrigo and Barker, 2012). Our findings indicate that the *C. × insueta* studied here might be in transition from a temporary hybrid derivative to a stabilized allopolyploid species. This plant displays genetic variation, shows efficient clonal growth and high vigour, has successfully spread across multiple microlocalities in the Unerboden valley (Fig. 1; see also Urbanska et al., 1997) and even promoted further hybridization by crossing with a non-parental species (*C. pratensis*), giving rise to a hybrid with a higher ploidy level, *C. × schulzii*. Although *C. × insueta* has reduced fertility (Mandáková et al., 2013), its multiple origins, coupled with occasional gene flow, can increase genetic variation and generate unique genotypes, thus ensuring long-term stability and evolutionary success.

In conclusion, this study describes a complex case of recurrent hybridization and polyploidization events resulting in multiple allopolyploids. This system and the findings that it has yielded call for further in-depth studies from various perspectives (evolution of duplicated genes, homeologue interactions, gene expression patterns, ecological divergence, etc.). We also suggest that revisiting earlier evolutionary hypotheses using more advanced molecular tools and approaches and larger population samples may significantly deepen our knowledge of the polyploid origins and evolution and can also bring completely new and exciting findings.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Marker Information: microsatellite marker development. Table S1: microsatellite markers and primers used in the present study. Table S2: genetic diversity measures for the sampled *Cardamine* taxa and populations. Table S3: allele-sharing patterns at selected microsatellite loci. Figure S1: neighbour-joining clustering of 266 individuals based on 10 *C. amara*-specific (Cama) microsatellite loci. Figure S2: principal coordinates analysis of 333 individuals based on six *C. rivularis* auct./*C. pratensis*-specific (Criv) microsatellite loci. Figure S3: neighbour-joining tree of 464 individuals based on four shared (C-all) microsatellite loci.

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- C. pratensis* L. s. str. ($2n = 4x = 32$, 1 ind.; $2n = 4x - 2 = 30$, 4 ind.; $2n \sim 4x$, 8 ind.), *C. rivularis* auct. non Schur \times *C. pratensis* L. s. str. ($2n = 5x - 2 = 38$, 1 ind.), *C. amara* L. subsp. *amara* ($2n = 2x = 16$, 8 ind.; $2n \sim 2x$, 54 ind.).
- Lej da Champfèr site** – CH, Graubünden, Lej da Champfèr, 1800 m, coll. KM and JŠ:
- C. amara* subsp. *austriaca* Marhold ($2n = 32$, $2n \sim 4x$, 9 ind.), *C. rivularis* auct. non Schur ($2n = 16$, $2n \sim 2x$, 15 ind.), *C. amara* subsp. *austriaca* \times *C. rivularis* auct. ($2n = 24$, $2n \sim 3x$, 15 ind.).

Comparative populations

Cardamine amara L. subsp. *amara*

Ste-Croix – CH, Vaud, Ste-Croix, 1209 m, coll. M. de la Harpe; **Wehrenbach** – CH, Zürich, Wehrenbach, coll. M. Helling; **Železná** – SK, Bratislava, Malé Karpaty Mts., Železná studnička, 270 m, coll. IK, $2n \sim 2x$; **Vrbice** – CZ, Podbořanská kotlina, Vrbice, 330 m, coll. IK, $2n \sim 2x$; **Hradišťany** – CZ, Plzeňská pahorkatina, Hradišťany, 400 m, coll. IK, $2n \sim 2x$; **Višňová** – CZ, Střední Povltaví, Višňová, 420 m, coll. IK, $2n \sim 2x$; **Telgárt** – SK, Telgárt, 890 m, coll. KM, ML, $2n \sim 2x$, 6 ind.; **Capatanii** – RO, Mt. Capatanii, Manastirei Pantrusa, 1000 m, coll. MP; **Osnabrück** – DE, Osnabrück, Sutthausen, 90 m, coll. K. Mummenhoff.

Cardamine amara subsp. *austriaca* Marhold

Flüelapass – CH, Graubünden, Flüelapass, 2380 m, coll. KM and JŠ, $2n = 32$; **Koralpe** – AU, Styria, Wolfsberg, Mt. Koralpe, 1960 m, coll. KM and JŠ, $2n = 32$.

Cardamine rivularis auct. non Schur

Valbella – CH, Graubünden, Valbella, lake Heidsee, 1480 m, coll. KM and JŠ, $2n = 16$; **Maloja** – CH, Graubünden, Maloja, lake Sils, 1810 m, coll. KM and JŠ, $2n = 16$; **Lej da Staz** – CH, Graubünden, St. Moritz, lake Lej da Staz, 1810 m, coll. KM and JŠ, $2n = 16$; **Weinebene** – AU, Carinthia, Wolfsberg, from Winebene to Grillitsch Hütte, 1720 m, coll. KM and JŠ, $2n = 16$; **Koralpe** – AU, Styria, Wolfsberg, Mt. Koralpe, 1960 m, coll. KM and JŠ, $2n = 16$; **Falkertsee** – AU, Carinthia, Nockberge, Falkertsee, 1940 m, coll. SŠ and MP, $2n = 32$; **Plannersee** – AU, Styria, Niedere Tauern, Planneralp, Plannersee, 1780 m, coll. SŠ and MP, $2n = 32$.

Cardamine pratensis L. s. str.

Trieben (P) – AU, Styria, Trieben, Hohentauern, 1065 m, coll. SŠ and MP, $2n \sim 4x$, 6 ind.; **Flattnitz** (P) – AU, Carinthia, Flattnitz, 1395 m, coll. SŠ and MP, $2n \sim 4x$, 6 ind.; **Vordernberg** (P) – AU, Styria, Vordernberg, 1080 m, coll. SŠ and MP, $2n \sim 4x$, 6 ind.; **Wegscheid** (P) – AU, Styria, Wegscheid, S of Mariazell, 910 m, coll. SŠ and MP, $2n \sim 6x$, 6 ind.

APPENDIX

Cardamine species and populations sampled in the present study. Taxon name, population code (in bold), country code (AU, Austria; CH, Switzerland; CZ, Czech Republic; DE, Germany; RO, Romania; SK, Slovakia), administrative division (canton, Bundesland), locality, altitude and collector name (IK, I. Krásná; JŠ, J. Šibík; KM, K. Marhold; ML, M. Lysák; MP, M. Perný; SŠ, S. Španiel) are given. Chromosome numbers ($2n =$) and/or DNA ploidy levels assessed by flow cytometry ($2n \sim$) are specified, either determined in the present study (in bold, providing also the number of analysed individuals) or taken from the literature (summarized in the database available at <http://www.cardamine.sav.sk> (accessed 3 September 2013)).

Urnerboden site – CH, Uri, Urnerboden valley, 1318–1380 m, coll. ML et al.:

C. \times insueta Urbanska ($2n = 3x = 24$, 32 ind.; $2n \sim 3x$, 60 ind.), *C. \times schulzii* Urbanska ($2n = 6x - 2 = 46$, 21 ind.; $2n = 5x - 2 = 38$, 6 ind.; $2n \sim 6x$, 5 ind.), *C. rivularis* auct. non Schur ($2n = 2x = 16$, 16 ind.; $2n \sim 2x$, 128 ind.),